# The olfactory responses of the antenna and maxillary palp of the fleshfly, *Neobellieria bullata* (Diptera: Sarcophagidae), and their sensitivity to blockage of nitric oxide synthase

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## Abstract

The relative sensitivities of the olfactory receptors in the antenna and maxillary palp of the fleshfly, *Neobellieria bullata*, were assessed using simultaneous electroantennograms (EAGs) and electropalpograms (EPGs). In general, the antennae and maxillary palps were more sensitive to odors related to animals (blood extract and saturated carboxylic acid) than to odors that were plant-derived (citral, hexenol, hexenal). In addition, the maxillary palps were relatively less sensitive to plant-derived odorants than the antennae, perhaps related to their anatomical position. Scanning electron microscopy was also used to assess the types of sensilla found on the two organs. In addition, NADPH-diaphorase histochemistry was used in an attempt to localize the enzyme nitric oxide synthase (NOS) in the antenna and the maxillary palps. We found evidence of NADPH-diaphorase staining in both organs, with localized staining in the antennal cells and more general staining in the maxillary palps. When NOS was selectively blocked using the antagonist L-NAME, the amplitude of the EAGs and EPGs to odorants fell by 30–50%. In contrast, application of the inactive enantiomer, D-NAME, did not change the amplitude of the EAGs or the EPGs. Our results indicate that NOS is involved in the function of olfactory receptor cells in the fleshfly.
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## 1. Introduction

For many insects, the chemical senses play a major role in decisions regarding feeding, mating and other behaviors. Among fleshflies, blowflies, and screwflies, a number of animal-related compounds and mixtures evoke either a physiological or behavioral response. For example, in the screwworm fly *Cochliomyia hominivorax* (Calliphoridae), dried bovine blood is an olfactory attractant in the field and in the lab (Hammack et al., 1989; Hammack, 1990), and artificial mixtures of animal-derived compounds such as swormlure-2 (Coppedge et al., 1977) are commonly used to monitor populations of screwworms in the field. The blowfly, *Phormia regina*, has shown behavioral sensitivity to aliphatic alcohols and aldehydes (summarized in Dethier, 1976). In our experimental animal, *Neobellieria* (previously *Sarcophaga*) *bullata*, a sarcophagid fly, larviposition behavior is affected by the presence of liver odor (Mitchell and Soucie, 1993).

Electroantennograms (EAGs) have been used to monitor physiological responses of olfactory receptors in a number of related fly species. In *Lucilia sericata*, receptors are sensitive to a wide variety of odorants, from $C_2$–$C_6$ alkyl thiols to cyclohexane, ethyl acetate and camphor, while in *Calliphora erythrocephala*, single receptor neurons respond to volatiles emanating from meat, carrion, and cheese, and to some alcohols, aldehydes and mercaptans (Dethier, 1976). In the stable fly (*Stomoxys calcitrans*), EAGs show responses to a wide variety of odors, including $CO_2$, cattle feces, human breath, acetone and 1-octen-3-ol (Schofield et al., 1995; Warnes and Finlayson, 1986). In comparison, Cork (1994) used extracts from sheep wounds infected with larval screwworms to assess the types and abundance of vol-

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atiles available to adult screwworms, and used EAGs to assess the effectiveness of the different odor components in stimulating the adult antenna. He found that the most abundant compounds in the wound odor were short-chain straight and methyl-branched aliphatic carboxylic acids, with the highest sensitivities in the EAGs to aliphatic carboxylic acids with 4–6 carbons; these compounds are typical of those found in mammalian secretory and excretory products.

In parallel, the evidence is strong that the gaseous messenger molecule nitric oxide (NO) and nitric oxide synthase (NOS), the enzyme that generates NO, play a role in insect olfaction as well as in other processes, including learning and memory consolidation; mechanoreception; visual processing; neural development; malpighian tubule function; and salivary gland function to facilitate blood feeding in *Rhodnius* (reviewed in Bicker, 1998, 2001; Davies, 2000; Müller, 1997). To date, in insects, NOS has been cloned from *Drosophila* (dNOS; Regulski and Tully, 1995); the hemipteran *Rhodnius prolixus* (Yuda et al., 1996); the sphinx moth *Manduca sexta* (MsNOS; Nighorn et al., 1998) and the mosquito *Anopheles stephensi* (Luckhart and Rosenberg, 1999).

In the olfactory organs, NADPH-diaphorase staining, indicative of the presence of NOS, has been reported in the antennae of *Apis* (Müller and Hildebrandt, 1995), *Drosophila* (Müller and Buchner, 1993), and *Manduca* (Stengl and Zintl, 1996), while in the locust, sensory afferents are not stained (Müller and Bicker, 1994; Bicker and Hähnlein, 1995; Elphick et al., 1995). In the antennae that were stained, the staining of the antennal cells was variable, ranging from the heavy staining of the olfactory neuron somata and axons in *Drosophila* (Müller and Buchner, 1993) to light staining in *Apis,* where the epithelial cells appeared to be more heavily stained than the afferents (Müller and Hildebrandt, 1995). In *Manduca*, Stengl and Zintl (1996) found variable staining in sub-populations of olfactory, mechano-, thermo- and hygroreceptors. They also found that NADPH-diaphorase staining of pheromone receptor neurons appeared to be correlated with exposure of the antenna to the sex pheromone.

Evidence from recent work in pupal *Manduca* using northern blots shows that NOS is expressed in the antennae (Nighorn et al., 1998), while in situ hybridizations and NOS immunocytochemistry show NOS expression in the olfactory receptor axons throughout pupal development, while its expression in the cell bodies of the receptor cells is transient. Staining of glial and other support cells in the antenna occurred at different points during development (Gibson and Nighorn, 2000).

Our study had several objectives, including the elucidation of the types of olfactory receptors found on the antennae and the maxillary palps of the fleshfly *Neobellieria bullata* (Fig. 1A) using scanning electron microscopy (SEM). We also assayed the differences, if any, in the olfactory sensitivities of the antennae and the maxillary palps to both single odorants and an odorant mixture. We hypothesize that based on their relative locations, the maxillary palps would perceive odors more directly involved in feeding while the antennae are more likely responsible for the perception of other chemicals, such as plant volatiles. We investigated this potential disparity through the use of simultaneous electroanntenograms (EAGs) and electropalpograms (EPGs). We tested the antennae and the palps with single, well-defined odorants including a saturated aliphatic acid, plant volatile components, and with a complex blood extract.

Another objective was to elucidate the possible role of NO in olfaction in these flies. We initially used NADPH-diaphorase histochemistry to investigate the putative distribution of NOS, the enzyme that produces NO, in the antennae and the maxillary palps. We then assayed the effects of the NOS blocker L-NAME on the olfactory responses of the antennae and the maxillary palps. As controls, we applied the inactive enantiomer D-NAME, as well as a saline solution.

## 2. Materials and methods

### 2.1. Animals

*Neobellieria bullata* were purchased as pupae from a commercial supplier (Carolina Biological Supply). Pupae were placed in a humidified plastic chamber at room temperature, and after eclosion, flies were starved but kept hydrated using Kimwipes (Kimberly-Clark) soaked in water.

### 2.2. Scanning electron microscopy

Air-dried heads of 2–3 day old *Neobellieria bullata* were sputter- coated with platinum and viewed using an ISI-40 Scanning Electron Microscope at the Ohio Agricultural Research and Development Center, Wooster, OH.

### 2.3. EAG and EPG recording techniques

EAG and EPG responses were simultaneously measured on adult male and female *Neobellieria bullata* using standard protocol (Roelofs, 1984). As Crnjar et al. (1990) have shown that age influences EAG response in *Phormia regina,* we used only flies 2–3 days post-eclosion for our assays. Individual flies were selected and anaesthetized by cooling in a refrigerator for approximately ten minutes. The fly was then strapped onto a specially constructed block of balsa wood with its head at the level of the top of the block by means of a thin strip of sticky dental wax (Kerr) placed across the back
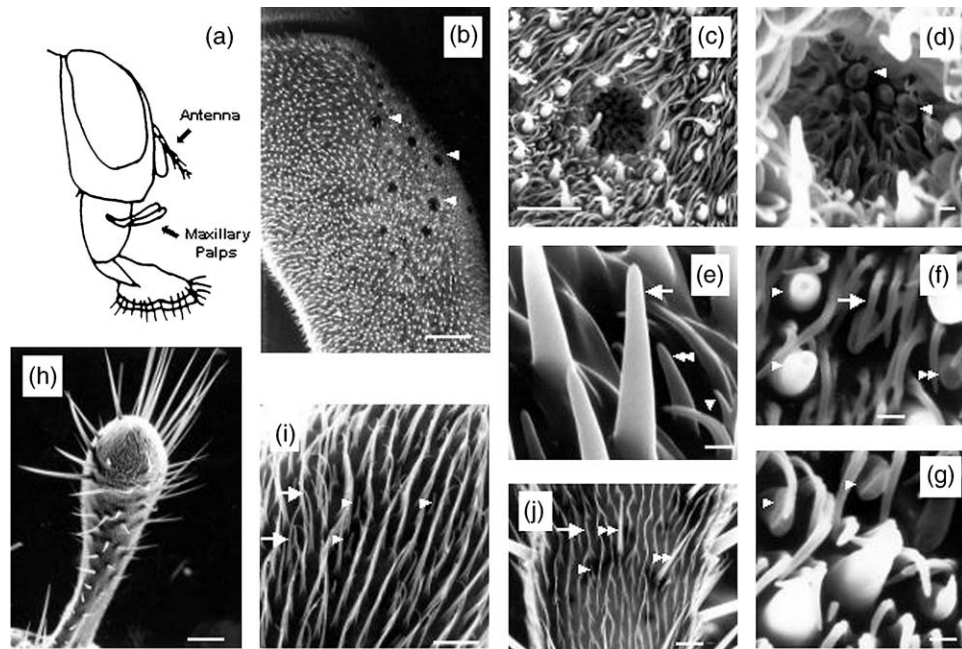
Fig. 1.   Diagram of a fly head and scanning electron micrographs of the antenna and the maxillary palp in *N. bullata*. A. Diagram of a fly head with the antenna and the maxillary palps (redrawn from Dethier, 1976). B. Dorsal view of the flagellum (3rd segment) of the right antenna. Note the pits on the medial surface (arrowheads). C. Close-up view of one of the pits. D. A view into the pit in C. Note the *sensilla basiconica* in the pit (arrowheads). E. A close-up view of the antenna near the distal tip. Note the trichoid sensillum (arrow); the microtrichia (single arrowhead); basiconic sensillum (double arrowhead). F. A close-up view of the antenna near the tip. Microtrichia (arrow) and basiconic sensillum (double arrowhead) as above. Note the two sensilla with what appear to be pores at the tips (single arrowheads). G. Novel flattened *sensilla basiconica* on the antenna near the tip. H. Overview of the maxillary palp. Note the long mechanosensory bristles. I. Close-up view of the maxillary palp near the tip. Note the basiconic sensilla (arrowheads) interspersed among the microtrichia (arrows). J. Close-up view of the maxillary palp, mid-shaft. Note the smaller bristles (double arrowheads); the microtrichia (arrow); and the basiconic sensillum (single arrowhead). Scale bars=50 µm (B,H); 10 µm (C,I, J); 1 µm (D,E,F,G).

of the fly. The fly was then hydrated with two-three drops of deionized water. The proboscis was held in an extended position on top of the block using a metal staple formed from a minuten pin. A thin silver wire coated with AgCl inserted into the eye acted as the reference electrode. One recording electrode, a broken-tipped glass micropipette filled with fly saline (NaCl 140 mM; KCl 5 mM; $CaCl_2$ 7 mM; $MgCl_2$ 1 mM; TES 5 mM; $NaHCO_3$ 4 mM; trehalose 5 mM; sucrose 100 mM, pH 6.9. N.J. Strausfeld, pers. comm.) was gently inserted into a small incision in the antenna made using a broken razor blade. A similar glass electrode was inserted into a small incision in one of the maxillary palps. EAGs and EPGs were amplified ten times using DC amplifiers (A–M Systems), further amplified using a homemade operational amplifier, displayed on an oscilloscope, and recorded onto tape using a modified VCR (Vetter Model 820, WinTron Technologies).

### 2.4. Stimulation procedures and apparatus

Stimuli were presented by means of an odor delivery system. The set-up consisted of two glass 1 cc syringes (Glaspak), one of which delivered a constant air flow (1 L/min) over the fly's antennae and maxillary palps at a distance of 2 cm from the preparation. The other syringe contained one of an array of odor stimuli, and was pointed at the fly head at about a 15° angle with respect to the constant air syringe and the same distance away from the preparation. Each odor syringe consisted of a rolled piece of filter paper (2 × 1.5 cm, Whatman #1), loaded with 10 uL of a given odorant solution (see below). The air was charcoal-filtered and humidified prior to delivery via the syringes. The stimulus was delivered by switching the airflow from the constant flow syringe to the odor stimulation syringe by the means of a solenoid-driven valve (General Valve), triggered by a physiological stimulator (Getting Instruments). Each stimulation lasted 1 s and a recovery interval of 3 min was allowed between each stimulation. During a given experiment, each animal received one stimulation of each odor in increasing concentration, with the order of the odor presentation within a given concentration being varied from day to day. The odor-saturated filter papers were replaced daily. A suction system powered by a muffin fan removed stimulus-containing air from the experimental area into a fume hood.

## 2.5. Test stimuli

We initially screened a larger set of odorants and dilutions than were later used for the study. We began with $10^{-1}$–$10^{-7}$ dilutions of butyric, valeric, and caproic acids (C4–C6 saturated carboxylic acids) and $10^{-1}$–$10^{-7}$ dilutions of the plant odorants citral, hexenal, and hexenol, all diluted in paraffin oil. In addition, we used a 10% dried blood mixture in paraffin oil and a blank of paraffin oil alone. The choice of the carboxylic acids was influenced by Cork (1994) who found that the C4–C6 aliphatic acids had high EAG activity in the screwworm fly. After the initial screening, we determined that the responses to the carboxylic acids were so similar that we used valeric acid in the body of the experiment to represent the short-chain aliphatic carboxylic acids found to be so effective as olfactory stimulants in the screwworm fly. As well, we found that odorant dilutions below $10^{-4}$ elicited little response, so we restricted the odorants to dilutions between $10^{-1}$ and $10^{-4}$. The following compounds were then used as the final suite of stimuli: citral, hexenal and hexenol (all from Sigma) represented typical plant odors; valeric acid (Sigma) and dried blood (Dragon brand, cow and pig blend) simulated the odors of flesh and blood to which *N. bullata* are attracted. All compounds were diluted in paraffin oil (Fisher) and presented in the dilution range $10^{-1}$–$10^{-4}$ from stock except for the dried blood, where we used 10 and 1% dried blood-paraffin oil solutions. Paraffin oil was the control in all experiments.

## 2.6. NOS pharmacology

To apply the pharmacological agents to the olfactory organs, the drugs were placed into the recording electrodes during the course of the experiment. Both N$^G$-nitro-L-arginine methyl ester (L-NAME) and its inactive enantiomer N$^G$-nitro-D-arginine methyl ester (D-NAME) were used (Müller, 1996; Zou et al., 1998). In the experimental treatment, 2 mM L-NAME (Sigma) in fly saline was substituted for the saline in the recording electrodes 15 min after the beginning of the experiment. In the controls, either 2 mM D-NAME (Sigma) or additional fly saline was substituted for the initial saline in the recording electrodes. These agents were applied for 20 min before the pipettes were refilled with saline. The antenna and maxillary palps were stimulated with valeric acid, 10% blood, and paraffin oil at 10 min intervals during the course of the 50 min experiment.

## 2.7. Analysis

Statistical analyses of the physiological data using ANOVA were performed using Minitab software (Minitab, Inc.) and graphs were constructed using Origin (OriginLab). EAG and EPG data were normalized for each animal by using the mean response to 10% dried blood in paraffin oil as 100% response.

## 2.8. NADPH-diaphorase histochemistry

For NADPH-diaphorase histochemistry, we modified the protocol of Elphick et al. (1995), running all the histochemical steps at room temperature. Briefly, isolated maxillary palps and antennae were fixed in fresh 4% paraformaldehyde in PBS (pH 7.4) in the refrigerator overnight, then washed $3 \times 15$ min in 50 mM Tris-HCl (pH 7.5), then incubated overnight at 4 °C in 2% Triton X-100 in 50 mM Tris-HCl. For the NADPH-diaphorase histochemistry, the tissues were reacted in 1mM NADPH and 0.5 mM Nitro Blue Tetrazolium (Sigma) in 50 mM Tris-HCl with 0.1% Triton X-100 in the dark until the tissue was stained, up to 2 h. Following a 15 min rinse in Tris-HCl with 0.1% Triton X-100 and 3 $\times$ 15 min rinses in Tris-HCl, the preparations were dehydrated and embedded in paraffin according to standard procedure (Presnell and Schreibman, 1997). 10 μm sections cut on a rotary microtome (American Optical) and mounted on albumen-coated slides. The slides were then deparaffinized, dehydrated in a graded ethanol series, cleared in xylene and coverslipped under Fluormount (Gurr).

## 2.9. Photomicrography and figure composition

The preparations were photographed using a Nikon Optiphot-2 microscope with Ektachrome 200 or Tri-X film (Kodak), processed, and the images digitized. The figures were composed in PowerPoint (Microsoft).

# 3. Results

## 3.1. Scanning electron microscopy

The scanning electron micrographs (Fig. 1B–J) show that the antennae and the maxillary palps are covered with sensilla. The flagellum of the antenna is dotted with a number of pits along the dorso-medial aspect that are lined with what appear to be basiconic sensilla (Fig. 1B–D, the terminology of Been et al., 1988). As well, the surface of the antenna is covered with microtrichia and trichoid sensilla and a variety of basiconic sensilla (Fig. 1E–G). Last, several sensilla that appear to have a terminal pore were also seen (Fig. 1F).

In the maxillary palps, the suite of sensilla was more restricted, with some noticeable variation in their distribution. The long mechanosensory bristles were the most obvious features (Fig. 1H, J), with a large number of microtrichia interspersed with basiconic sensilla. The basiconic sensilla were more numerous toward the tip of

the maxillary palp as compared to the shaft (compare Fig. 1I vs J).

## 3.2. EAG and EPG responses to different odors

All of the tested odors except for the paraffin oil, the odorant carrier, elicited electrophysiological responses (Fig. 2A, B), ranging in amplitude from 0–1.9 mV for the EPGs and up to 2.6 mV for the EPGs. In both the EAGs and EPGs, the 10% dried blood in paraffin oil elicited the greatest responses (mean amplitude for EAGs from all individuals=1.1 mV; mean amplitude for EPGs from all individuals=1.6mV). The mean response of each individual to 10% dried blood was defined as 100% response for the analysis of the other odors. In
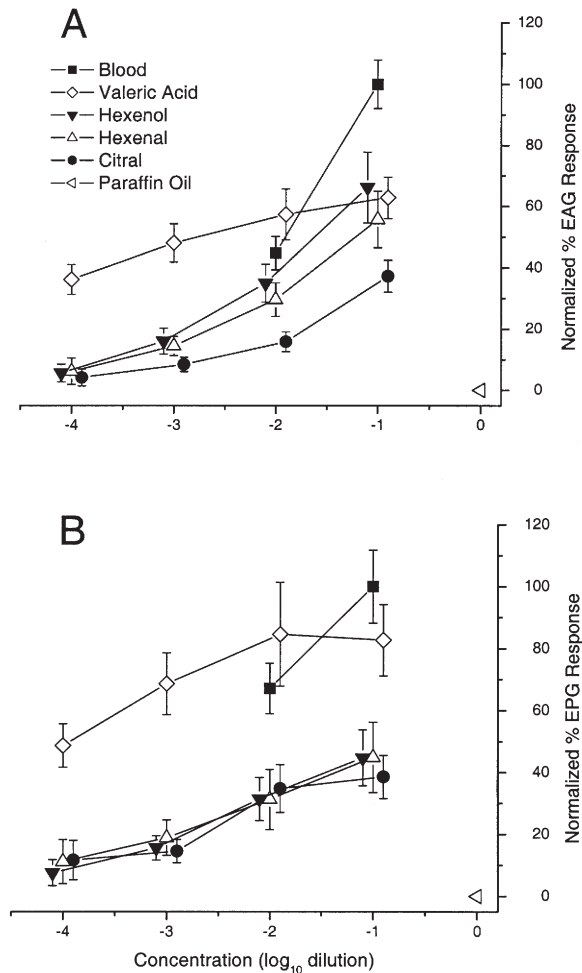


Fig. 2. EAG and EPG responses to various odorants in *N. bullata*. A. Mean (±SE) normalized EAG responses for different odorants (response to 10% dried blood=100% response). (ANOVA∗—Stimulus type, F = 64.76, p < .001; Stimulus dose, F = 16.64, p < .001). B. Mean (±SE) normalized EPG responses for different odorants (response to 10% blood=100%). (ANOVA∗—Stimulus type, F = 61.98, p < .001; Stimulus dose, F = 18.73, p < .001). For both A and B, n = 16 (blood), 43 (citral), 40 (hexenal), 37 (hexenol), 41 (paraffin oil), 37 (valeric acid). ∗ Due to the unbalanced data sets, interaction effects between the stimulus type and dose could not be calculated.

both the EAGs and the EPGs, the response to the dried blood did not plateau at the higher concentration of 10%, suggesting that higher stimulation levels would have elicited greater responses. Valeric acid had the highest activity at the lowest concentrations, showing a different, flatter, response curve than the other odorants in both the EAGs and the EPGs. From the lowest to the highest concentration, a 1000-fold change in the concentration of valeric acid resulted in the EAG and EPG response rising only by about 50%, as compared to the 4–6-fold increases in EAG and EPG responses to the single components of plant odors (citral, hexenal, hexenol) over the same concentration range. With the EPGs, the three plant odorants were not different in their responses over the entire concentration range. In contrast, in the EAGs, the responses were identical at the lowest concentration, while at the higher concentrations the response to citral became significantly lower those that of hexenal and hexenol. The relative responses to hexenal and hexenol were higher in the EAGs than the EPGs, with the slopes of the response curves increasing in the EAGs at the higher concentrations, while in the EPGs, the slope was stable or even beginning to plateau (see the EPG response to citral at $10^{-1}$, Fig. 2B).

## 3.3. NADPH-Diaphorase histochemistry

The staining in the antenna using NADPH-diaphorase histochemistry was restricted to the sensilla underlying the pits (Fig. 3A), most likely the basiconic sensilla shown in Fig. 1D. In the maxillary palps, the staining was widespread, with the staining being most prominent toward the distal tip (Fig. 3B).

## 3.4. EAG and EPG responses to L-NAME and D-NAME

The EAG and EPG amplitudes fell by 30–50% in response to the application of the NOS antagonist L-NAME to the antenna and the maxillary palps, while the application of the inactive enantiomer D-NAME or saline had little or no effect (Fig. 4A–F). The EPG response to blood fell faster in the presence of L-NAME than the EAGs, showing a strong decrease within 5 min of the application, while the EAG responses initially fell somewhat more slowly. In both olfactory organs, the replacement of the L-NAME filled electrode with a saline-filled electrode did not result in a reversal of the decline in the responses during the remainder of the 50 min experiment.

## 4. Discussion

### 4.1. Morphology

Our morphological work shows that the sensilla found on the antenna and maxillary palps of *Neobellieria bul-*
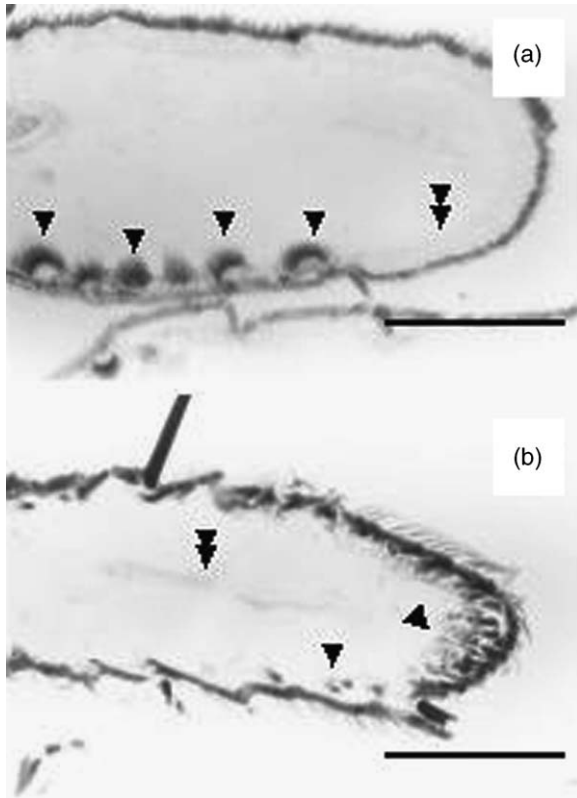
Fig. 3. NADPH-diaphorase histochemistry of olfactory organs of *N. bullata*. A. Longitudinal section of the antenna. Note the heavy staining in the cells associated with the antennal pits (single arrowheads) and lack of staining in the other parts of the antennal epithelium (double arrowhead). B. Longitudinal section of the maxillary palp. Note the heavy staining especially at the distal tip and in some of the other epithelial cells (single arrowheads). The maxillary nerve is unstained (double arrowhead). Scale bars=200 μm (A) or 100 μm (B).

*lata* are similar to those found in other flies. Among flies, most is known about olfaction in *Drosophila* due to its genetic and developmental advantages. As in other flies, the *Drosophila* antenna consists of three antennal segments, with the third segment, the funiculus (or the flagellum), being the most prominent and having a prominent bristle, the arista, arising from it. The funiculus is also the location of all of the known olfactory receptors on the antenna (Stocker, 1994). Detailed studies have shown that there are several different types of olfactory sensilla on the funiculus: *sensilla basiconica* (single-walled, multi-porous); *sensilla coeloconica* (double-walled, wall pored); and *sensilla trichoidea* (single-walled, pored?) which may or may not have olfactory function (Riesgo-Escobar et al., 1997b; Stocker, 1994). Similar sensilla are found on the antenna of other flies. In the Queensland fruit fly (*Bactrocera tryoni*), Hull and Cribb (1997) found six types of sensilla on their antenna: *sensilla chaetica*; two types of *sensilla trichoidea*; *sensilla basiconica*; sensilla *styloconica*; clavate sensilla; along with uninnervated microtrichia. In the antenna of *Phormia regina* and in *Musca autumnalis*,

Dethier (1976) has shown a variety of basiconic and grooved styloconic sensilla. Been et al. (1988) working with the Sheep head fly (*Hydrotaea irritans*), found olfactory sensilla only on the funiculus of the antenna, categorized into *sensilla trichoidea* (thick-walled, multiporous sensilla), *sensilla styloconica* (double-walled, grooved sensilla), and six types of *sensilla basiconica* (thin-walled, multiporous sensilla).

In addition, maxillary palps of some insects play an olfactory role. In flies, there are *sensilla basiconica* on the maxillary palps whose structures are similar (*Drosophila*—Singh and Nayak, 1985; *Calliphora*—van der Starre and Templaar, 1976) or dissimilar (*Hydrotaea*—Been et al., 1988) to those on the antenna. In *Drosophila*, Singh and Nayak (1985) have also found *sensilla trichoidea* as well as "spinules" that are spatially segregated on the surface of the maxillary palp. In other flies, van der Starre and Templaar (1976) found long mechanoreceptive hairs in addition to the basiconic sensilla in *Calliphora*, while Been et al. (1988) found microtrichia and "socketed" bristles along with the basiconic sensilla in *Hydrotaea*. Physiologically, the maxillary palps have also been shown to respond to a variety of odorants in *Drosophila* (Ayer and Carlson, 1992; de Bruyne et al., 1999; Riesgo-Escobar et al., 1997a) and in *Calliphora* (van der Starre and Templaar, 1976).

In *Neobellieria*, the surface of the antennal flagellum is covered with microtrichia and sensilla with probable olfactory function, including trichoid sensilla and a variety of basiconic sensilla, including some that have a flattened morphology (Fig. 1G), similar to those seen in *Phormia regina* (Dethier, 1976). Several other types of basiconic sensilla were also observed, including some that have a small protrusion at the tip (Fig. 1D,G), and others that are more conical or cylindrical in shape (Fig. 1E,F). These are among the types categorized by Been et al. (1988) in the muscid fly, *H. irritans*. Interestingly, we saw no examples of styloconic sensilla, the double-walled sensilla with grooved exteriors that have been seen in *P. regina* and *M. autumnalis* (Dethier, 1976); *Bactrocera tryoni* (Hull and Cribb, 1997); *H. irritans* (Been et al., 1988); and *Drosophila* (Stocker, 1994; Riesgo-Escobar et al., 1997b—called coeloconic sensilla in the *Drosophila* literature). Last, we saw near the tip of the antenna several examples of sensilla with what appeared to be pores at the tips (Fig. 1F). These may be gustatory sensilla, as they have morphology similar to those found on the mouthparts of other flies (Dethier, 1976).

Invagination or pits in the antenna (called the sacculus in *Drosophila*) are a feature common to fly antennae, found in *Drosophila* (Stocker, 1994); *H. irritans* (Been et al., 1988); and *M. autumnalis* (Dethier, 1976), typically on the dorso-medial aspect of the antenna. Basiconic sensilla are features of the pit in *Drosophila* and *Musca*, similar to what we see in *Neobellieria*, while the
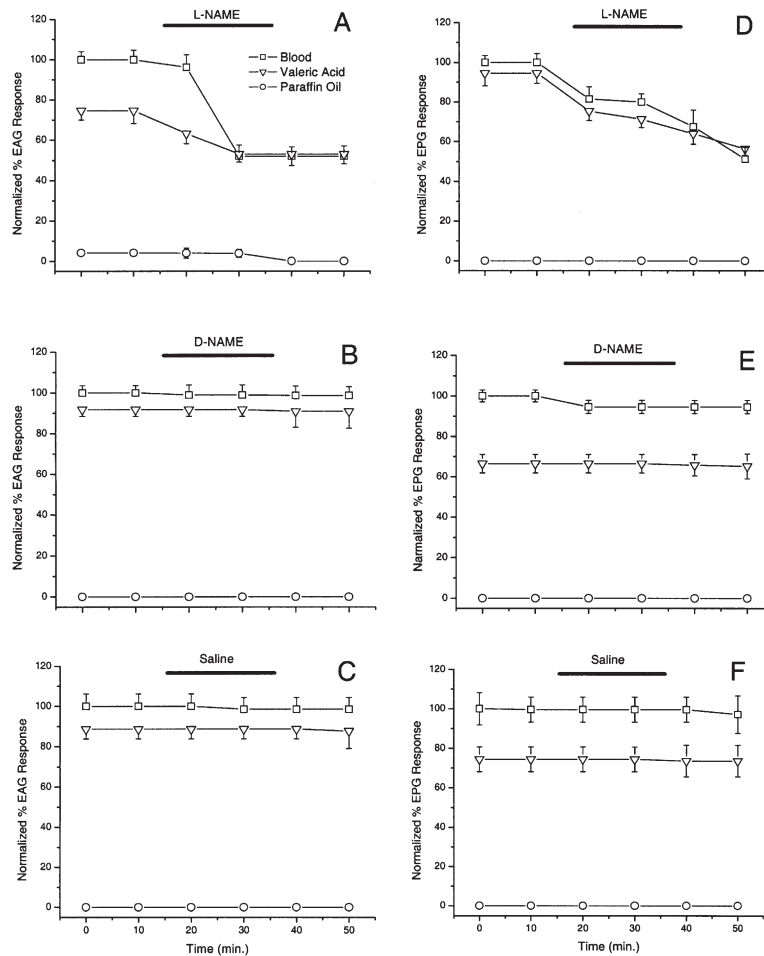
Fig. 4. EAG and EPG responses (mean±SE) following treatment with the NOS antagonist L-NAME, the inactive enantiomer D-NAME, or saline. All responses normalized to the mean initial response to 10% dried blood (response to 10% dried blood=100%) for that experiment. Dark line indicates time of application of L-NAME, D-NAME, or saline. A. EAG response to blood, valeric acid, and paraffin oil in the presence of L-NAME. B. EAG response to blood, valeric acid, and paraffin oil in the presence of D-NAME. C. EAG response to blood, valeric acid, and paraffin oil in the presence of saline. D. EPG response to blood, valeric acid, and paraffin oil in the presence of L-NAME. E. EPG response to blood, valeric acid, and paraffin oil in the presence of D-NAME. F. EPG response to blood, valeric acid, and paraffin oil in the presence of saline. n = 7 for all values.

sensory complement of the pit in *Hydrotaea* is not known.

The maxillary palps in *Neobellieria* are less complex, with sensilla essentially identical to those found in *Drosophila* (Ayer and Carlson, 1992; de Bruyne et al., 1999; Stocker, 1994), Calliphora (van der Starre and Templaar, 1976), and *Hydrotaea* (Been et al., 1988). They consist of microtrichia and socketed mechanosensory bristles intermingled with what appeared to be only one type of basiconic sensilla. Been et al. (1988) also observed the same non-random distribution of the basiconic sensilla as we did, finding that they were concentrated in the distal portions of the maxillary palps in *Hydrotaea*.

### 4.2. EAGs, EPGs and olfactory function

Both the antennae and the maxillary palps were stimulated by the odorants we used (Fig. 2). Single compo-

nents of plant odors including, citral, hexenal, and hexenol yielded greater relative responses from the antennae than from the maxillary palps. In the antenna, there were consistent differences in sensitivity to these plant odors, with the hexenal and hexenol consistently eliciting slightly higher responses than the citral at the same dilutions, although the differences may be due to differences in volatility rather than receptor sensitivity. In contrast, the amplitudes of the EPGs did not differ between these three plant odors across all of the dilutions. One interpretation of the differences in response to citral may be that the maxillary palp is relatively more sensitive to citral than the antenna when compared to hexenal and hexenol. The differences between the EAGs and EPGs are not likely to be due to differences in odorant presentation, as both organs were simultaneously stimulated from the same odor cartridge; the short distance between the two organs make it unlikely that they received different amounts of odorant from the same cartridge.

Valeric acid elicited the highest responses at the lowest concentrations in both the antenna and the maxillary palp, with both dose-response curves coming to an apparent plateau at the highest concentrations. In contrast, the physiological response to the blood mixture did not appear to approach a plateau at the higher concentration, with both the antenna and the palps exhibiting the strongest response to the blood mixture at the higher concentration. This is likely due to the fact that the different components of the odorant mixture stimulated different receptors with different sensitivities, so that many different types of receptors were stimulated. In comparison, fewer receptor types are likely to be stimulated by single odorants, resulting in a weaker relative response and easier saturation or habituation of receptors. In sum, odors associated with meat produced the largest responses from both the antenna and the maxillary palps, however, the antenna showed higher relative sensitivity to the plant-related compounds than the palps. These data suggest that a degree of functional specialization exists between the two organs.

Our data from *Neobellieria* are in line with the results from previous studies, and show that this fleshfly is capable of detecting both plant and animal-derived odors. Ayer and Carlson (1992), using EAGs and EPGs, showed both organs in *Drosophila* responded to acetone, propionic acid, benzaldehyde, butanol, and ethyl acetate. There was a greater response to all the odorants from the antenna in comparison with the maxillary palps, although within each olfactory organ, the relative sensitivities to each odorant was different. In other flies, a large number of odorants activate the antenna, including $C_2$–$C_6$ alkyl thiols, cyclohexane, ethyl acetate, and camphor (*Lucilia sericata*—Dethier, 1976); meat, carrion, cheese odors and some alcohols, aldehydes and mercaptans (*Calliphora erythrocephala*—Dethier, 1976); $CO_2$, acetone, 1-octen-3-ol, and cattle feces (*Stomoxys calcitrans*—Warnes and Finlayson, 1986); and straight and methyl-branched short-chain aliphatic carboxylic acids (*Cochliomyia hominivorax*—Cork, 1994).

### 4.3. NADPH-Diaphorase histochemistry and NOS pharmacology

We used the easy NADPH-diaphorase histochemistry to assay the putative distribution of NOS, as NADPH-diaphorase activity has been co-localized with NOS (Hope et al., 1991). The patterns of NADPH-diaphorase staining in the antenna and the maxillary palps of *Neobellieria* are quite different, with the antenna showing very localized staining in the cells associated with the pits on the antennal surface, while the maxillary palps showed more general staining, especially toward the distal tip. The basiconic sensilla located in the antennal pits are most likely olfactory, and the cells associated with these sensilla is stained. In the maxillary palps, a larger suite of cells is stained. Variability in NADPH-diaphorase staining in the olfactory organs has been seen before in insects, with heavy staining in the olfactory cells and axons in *Drosophila* (Müller and Buchner, 1993), and light staining in *Apis,* with the antennal cells more heavily stained than the axons (Müller and Hildebrandt, 1995). In *Manduca*, variable staining was found, with a sub-population of olfactory, mechano-, thermo- and hygroreceptors showing activity (Stengl and Zintl, 1996). Müller (1997) suggested that the variability of the staining patterns in the antenna among species indicates distinct, specific roles for NO, rather than an evolutionarily conserved role for NO in chemosensory transduction.

More recent work has cast doubt on the utility of NADPH-diaphorase as a marker for NOS in some orthopterans (Ott and Burrows, 1999) and in *Manduca* (Nighorn et al., 1998; Gibson and Nighorn, 2000). In insect antenna, one alternative explanation is that the NADPH-diaphorase staining is related to the degradative activity of NADPH-cytochrome P450 oxidoreductase rather than to the activity of NOS (Nighorn et al., 1998; Bicker, 2001). NADPH-cytochrome P450 oxidoreductase is expressed in *Drosophila* antenna, where it may function to clear odorants from around the receptor cells (Hovemann et al., 1997).

In our experiments, the application of L-NAME, a nitric oxide synthase inhibitor, decreased substantially the EAG and EPG responses to blood and valeric acid. The application of D-NAME, the inactive isomer, or saline, had no effects on the EAG and EPG responses, suggesting a major role for NO in these flies in some part of the olfactory transduction or signal conduction process. Recent work in pupal *Manduca* using northern blots show that NOS is expressed in the antennae, and that soluble guanylyl cyclase (sGC), the major target for NO, is highly expressed in the antennal lobe (Nighorn et al., 1998). In comparison, in situ hybridizations and NOS immunocytochemistry show that NOS is expressed in the olfactory receptor axons throughout pupal development. Interestingly, NOS expression in the cell bodies of the receptor cells is transient, while glial and other support cells in the antenna at also stain for NOS at different points during development (Gibson and Nighorn, 2000). In adult *Manduca*, Stengl et al. (2001) have assayed sGC activity using cGMP immunocytochemistry, and have shown that a subpopulation of the sex pheromone-sensitive olfactory cells in the male antenna respond to the presence of both NO and sex pheromone by increasing cGMP levels. Aside from this potential role of NO and NOS in pheromone perception, NO is believed to play a developmental role in the antennal lobe, as NOS is highly expressed in the antennal nerve during pupal development while sGC expression and cGMP levels are elevated in parallel in some antennal lobe neurons (Schachtner et al., 1999; Gibson and Nighorn, 2000).

Interestingly, work in vertebrates show direct effects of NO in the olfactory transduction pathway that does not require sGC and cGMP. In the tiger salamander olfactory receptor cell, single channel recordings show that a cyclic nucleotide-gated channel is activated by NO (Broillet and Firestein, 1996), while in the rat olfactory cell, NO inhibits a similar cyclic nucleotide-gated cation channel (Lynch, 1998). In contrast, in the anurans *Xenopus laevis* and *Caudiverbera caudiverbera*, NO activates a K$^+$ channel in olfactory receptor cells (Schmachtenberg and Bacigalupo, 1999). While we cannot narrow the role of NO and its mechanism in insect olfaction from our work, it is clear that it plays a substantial role, quite possibly in the transduction process.

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